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Hybridomas Derived from Murine Gut Associated Lymphoid Tissue

Robert Staszewski

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ROBERT STASZEWSKI

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Robert Staszewski
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ABSTRACT

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The clinical potential of specific passive mucosal immunity is discussed and it is proposed that bulk quantities of hybridoma produced specific dimeric IgAs could be clinically useful as made to order super colostrums.

sIgAs function on mucosal surfaces to prevent the adherence of pathogens. They are poor opsonins and do not activate complement except by the alternate pathway.

In principle mesenteric lymph node (MLN) or Peyer's patch (PP) lymphocytes aseptically harvested from enterically immunized and boostered mice displaying blastogenic anamnestic response might be fused with log phase cells of a non secreting murine myeloma to generate specific dimeric IgA(+J) secreting hybridomas.

Lipoteichoic acid (LTA) has been demonstrated to be the adhesin of the group A streptococci and possibly many other gram positive mucosal pathogens. The polyglycerolphosphate backbone common to all LTAs is a 25-30 residue polymer affording many identical binding sites to a single specificity (monoclonal) antibody. Preincubation of streptococci with backbone specific antibody has been shown to block adherence

to human buccal epithelial cells in vitro.

Murine hybridoma derived dimeric IgA anti LTA backbone will likely have considerable ability to block adherence of streptococcal pathogens in vitro. In vivo it may be useful as a safe clinical agent providing passive mucosal immunity against a wide variety of gram positive pathogens, and might indeed be employed as a super colostrum.

A step toward this goal was taken by demonstrating IgA secreting murine hybridomas can be produced using gut associated lymphoid tissue (GALT) from enterically immunized and boostered animals.

MLN and PP lymphocytes from two groups of 5 mice intragastrically immunized and boostered with Freund's complete adjuvant (FCA) were separately pooled and fused with SP2 cells.

MLN cells were found to be poor fusion partners with a hybridization frequency 1/10 that of PP or spleen with SP2 cells, paralleling the 1:10 concentration ratio of surface immunoglobulin bearing blasts in these tissues.

The 20 day post fusion supernatants of all 12 MLN hybrids and 25 of the 35 (71%) of PP origin failed to demonstrate antibody production on ELISA. The difference is significant at $P=0.1$, $X_c^2(1df)=2.8$, and it is conjectured these MLN blasts were at a stage of differentiation less adept than PP blasts at promoting antibody secretion in

SP2 hybrids.

The observation of 4 IgA, 2 IgG, and 4 IgM containing supernatants is consistant with the 54:16:30 proportion of PP blasts displaying these surface antibodies, X_c^2=.185.

No attempt was made to determine antibody specificities as FCA was assumed to produce generalized GALT stimulation.

Work is currently in progress to assay the IgA containing supernatants for polymeric antibody.

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INTRODUCTION

THE CLINICAL POTENTIAL OF PASSIVE MUCOSAL IMMUNITY

The neonatal immune system is naive. The ignorance of the systemic and mucosal systems is due to their inexperience with environmental antigens.

Maternally derived passive immunity is provided through transplacental transfer of IgG and the ingestion of secretory IgA (sIgA) in colostrum. This sIgA is uniquely suited to passively protect the infant gut from enteric pathogens that the mother was recently exposed to and represent immediate threats till the infant gut associated lymphoid tissue (GALT) can produce enough sIgA to defend itself (1,2,3).

In principle passive immunity against mucosal surface pathogens can be provided by the application of specific sIgA to the surface as in the treatment of selective IgA deficiency with colostrum (4,5,6). Colostrum is difficult to obtain and the sIgA of uncertain antigenic specificity profile.

If it were available specific dimeric IgA even of heterologous origin might safely be used to develop agents to:

1) Treat sIgA deficiency states by ingestion or inhalation (4,5).

2) Use against the development of allergies in children of atopic parents by blocking absorption of potential allergens (7).

3) Provide specific short term prophylaxis or treatment for:

a) epidemic enteric illness by neutralizing virus or preventing the adherence of bacterial pathogens to mucosal surfaces, e.g. rotavirus and cholera in the gut, Neisseria meningitidis colonization of the oropharynx, Streptococcus mutans adherence to teeth, or group A streptococci adherence to pharyngeal mucosa (8,9,10,11,12).

b) pathogens on extraenteric mucosal surfaces, e.g. organisms causing otitis media, Neisseria gonorrhea or chlamydia adhering to urethral, vaginal, or conjunctival surfaces (11,13).

4) Provide the basis for a new class of contraceptives which might work by:

a) preventing the adherence of sperm to ovum (14).

b) preventing the adherence of the zygote to uterine mucosa (14,15).

MONOCLONAL ANTIBODIES IN ANY AMOUNT

In recent years bulk production of monoclonal antibodies, antibodies of a single predetermined specificity, has become possible by immortalizing lymphocyte lines via SV40 and Epstein Barr virus transformation or creating somatic hybrids of B cells and myeloma of mice or rats (16,17,18,19). It is even possible to produce human/mouse somatic hybrids which secrete human immunoglobulin (20).

Hybrids are produced when short lived B cells derived from lymph node or spleen of an animal displaying anamnestic response to rechallange with antigen are fused with polyethylene glycol or Sendai virus with cells of a non secreting myeloma (19). Non immunoglobulin producing murine myelomas currently in use (X63-Ag-8.653, Sp2/0-Ag-14, and F0) are all descended from the IgG_1, k secreting mineral oil plasmacytoma 21, MOPC 21 (22,23).

Unfused B cells die within days without proliferating but hybrid and myeloma clones have unlimited ability to proliferate. Selecting the few hybrids from the very large number of myeloma clones would be difficult if not impossible so the pioneers of the technique devised systems ensuring the selective survival of the somatic hybrids.

The novel technique of Wright allows for isolating heterokaryons between any two types of cells by treating

each with a lethal dose of an irreversible biochemical inhibitor of complementary specificity (iodoacetamide or diethylpyrocarbonate) (18). Only heterokaryon fusomas recieve full sets of unpoisoned molecules, half from each parent.

The technique of Kohler and Milstein is based on the use of a selective culture medium and is the current standard (19). Hypoxanthine guanine phosphoribosyl transferase (HGPRT) is the critical enzyme in the mammalian purine salvage pathway, myeloma cells lacking the enzyme must synthesize all purines de novo. $HGPRT^-$ myeloma cells are cultured in media containing the antimetabolite 8 azaguanine before fusion to prevent contamination with $HGPRT^+$ revertant clones. Azaguanine is toxic to normal cells because it is salvagable as a purine analog and incorporated into DNA, rendering it untranscribable.

Aminopterin is a folate antagonist which blocks both de novo purine synthesis and the methylation of deoxyuridylate to thymidylate. $HGPRT^-$ myeloma cells cannot grow in the presence of aminopterin even if thymidine and a salvagable purine like hypoxanthine are supplied (HAT medium) since salvage is impossible without HGPRT and synthesis is blocked by aminopterin.

Hybrids of B cells and $HGPRT^-$ myeloma cells are at least temporarily $HGPRT^+$, can salvage hypoxanthine and proliferate in HAT. Surviving hybrids may be

subcloned and tested for production of specific antibody by various means including radioimmunoassay (RIA), or enzyme linked immunosorbent assay (ELISA) (24). Promising clones can be grown in bulk culture or stored frozen. Antibody can be recovered from culture supernatant in a chemostat and purified via ammonium sulfate precipitation or cells may be injected intraperitoneally into MHC compatible mice and antibody rich ascites (containing 10-100 times the concentration in supernatants) harvested (19,20,25).

Up to now the technology has focused on the production of monoclonal IgG and IgM against viral, bacterial, murine or human cell surface antigens for use as laboratory investigational agents and very recently for the experimental diagnosis and treatment of several human malignacies (26,27).

In principle B cells obtained from tonsils, spleen, mesenteric lymph nodes (MLN), intestinal lamina propria, or Peyer's patches (PP) of an enterically immunized mouse could be fused with cells of a non secreting murine myeloma to investigate B cell differentiation in the GALT or to produce bulk quantities of specific dimeric IgA for clinical use.

THE MUCOSAL IMMUNE SYSTEM

We are under siege without and within. Bacteria, virus, protozoa, fungi, parasites, non replicating agents, and neoplasms would overrun us but for humoral immunity (B cell), cell mediated immunity (T cell), phagocytosis and complement. Our defenders may turn on us if they mispercieve self as foreign.

Secretions bathing mucosal linings and mechanical factors (skin and ciliary clearance) are among the first lines of defense against the external environment. The observations that antibody levels in mucosal fluids correlated more directly with resistance to certain infections than did serum titers, the preponderance of IgA in these fluids (IgA/IgG greater than 1), that IgA, IgM, and IgE are present in larger amounts than can be explained by simple transudation from serum (where IgG/IgA greater than 4), and that the antibody response in secretions was independent of the serum response led to the concept of a secretory immune system (28,29,30,31).

It is now known that the immunoglobulins in external secretions are produced by plasma cells locally in the lamina propria of the mucous membranes of the gut, nasal mucosa, salivary, lacrimal, bronchial, and mammary glands (32,33). In the presence of active inflamation they are supplemented with serum proteins

and IgG due to increased capillary permeability.

IMMUNOGLOBULIN STRUCTURE

The basic immunoglobulin monomer is a B lymphocyte or plasma cell product composed of four polypeptide chains. One pair of identical heavy chains (H chains) approximately twice the molecular weight of the light chains (L chains) (34).

There are five serologically determined classes of H chain, A, G, M, E, D, but there are only two such L chain classes, Kappa and Lambda (34,35). Immunoglobulin classes are designated by there respective heavy chain markers as IgA, IgG, IgM, IgE, and IgD.

The chains are folded into variable and constant domains by tertiary forces and disulfide bonds, and are held together by noncovalent forces and interchain disulfide bridges to form a bilaterally symmetric structure resembling a lobster.

H chain domains are labeled V_H, C_{H_1} - C_{H_4}, those on the L chains V_L and C_L. The antigen binding site, specificity, and serologic idiotype is formed in the V regions of the H and L chains. The area between C_{H_1} and C_{H_2} (the hinge region) is relatively flexible, allowing the lobster to reach out its claws for antigen.

IgA STRUCTURE

Human serum IgA exists mainly as a 7S monomer with 10-15% in polymeric form, and can be subdivided into two subclasses by serological H chain markers (30,36, 37,38). IgA_1 comprises 90% of serum IgA and marrow plasma cell product while IgA_2 makes up 40-60% of the immunoglobulin content of secretions.

IgA_2 is subdivided by allotypic H chain markers into an IgA_{2M_1} without L-H disulfide bonds, prevalent in caucasians, and an IgA_{2M_2} prevalent in mongoloid and negroid peoples.

These antigens are responsible for the anaphylactic and urticarial transfusion reactions encountered in IgA deficient patients (39,40).

Pathogenic Neisseria gonorrhea and meningitidis produce an IgA protease which cleaves a single proline-threonine bond in the hinge region of the IgA_1 A chain rendering the molecule unable to agglutinate bacteria (13).

SECRETORY IgA STRUCTURE

Secretory IgA (sIgA) the preponderant immunoglobulin in secretions, is a complex molecule composed of an IgA dimer (MW 300K), a molecule of J chain (MW 15K), and a molecule of secretory component (SC) (MW 70K) (35,41).

J chain is a plasma cell derived glycopeptide with

heavy glutamic and aspartic acid content, disulfide bonded to dimeric IgA at the penultimate carboxyterminal cysteine of the A chain, and not found associated with monomeric IgA (41,42,43). Complexing with J chain alters the conformation of the subunits bringing them close enough for intersubunit disulfide bond formation and also confering the ability to bind SC (41).

SC, an epithelial cell derived glycoprotein, acts as a receptor for dimeric IgA+J or pentameric IgM+J on the epithelial cell surface facing lamina propria (44). SC initiates the pinocytosis of the dimeric IgA+J that has diffused through the interstitium of the lamina propria to the epithelial intercellular space and ensures its transport onto the mucosa.

SC coils about the Fc portion of the dimer hinge region to hinge region, stabilizing it and increasing its resistance to proteolysis (41,44). SC has an affinity for mucin thereby giving sIgA a selective advantage to clear sIgA-antigen complexes by way of the constantly excreted mucin.

SC is present free in normal mucosal fluids as well as in the secretions of neonates (who normally have no detectable IgA) and in patients with selective IgA deficiency or agammaglobulinemia.

Polymeric IgA and polymeric IgA-antigen complexes are rapidly cleared from plasma by transhepatic transport into bile in mice and rats (45,46,47,48,49). It has

been demonstrated that dimeric IgA(+J) binds specifically to SC on human hepatocyte membranes in vivo, but that only 1% of an injected dose of polymeric antibody is recovered in bile in 8 hours (45,50). Transhepatic clearance of polymeric IgA and polymeric IgA antigen complexes is of minor importance in humans (50).

Several reports in the literature indicate human SC can also bind heterologous polymeric immunoglobulins including murine polymeric IgAs (51,52).

B CELL DIFFERENTIATION

Stem cells appear in the fetal liver and travel to the marrow (34,35). In that microenvironment some mature to become short lived B cells, lymphocytes characterized by immunoglobulin on their surfaces. Stem cell maturation and antibody production are active throughout life (34,35).

Antibody on B cell membranes is synthesized by that cell, IgM and IgD initially appearing simultaneously. These thousands of surface IgM and IgD molecules act as receptors for a single preordaned antigenic determinant. B_M cells rapidly populate the spleen and central nodes (B_M cells synthesizing J chain congregate in the lamina propria and draining nodes of secretory epithelia), where encounter with antigen will cause some to differentiate into IgM secreting plasma cells, a primary immune response.

Some will accept a T cell or macrophage signal and change the class, but not the specificity of its surface receptors, to IgG to become B_G cells. Some B_G cells encounter their antigen, proliferate and differentiate into IgG plasma cells, a secondary immune response. Some will accept a T cell signal and further differentiate into B_A cells with surface IgA, again to proliferate and differentiate to IgA plasma cells in response to antigen.

IgE producing cells seem to arise directly from B_M cells via T cell influence.

sIgA SECRETING TISSUES

Fluorescent antibody techniques show the bulk of sIgA production occurs in secretory tissues (38). IgA producing submucosal plasma cells in the human GI tract outnumber IgG producers 20-30:1 vs peripheral lymph nodes and spleen where the ratio is 1:5 (38). IgM and IgE in secretions are also local products but there are far fewer plasma cells of these classes. IgG in most external secretions is largely a non specific serum transudate. The lamina propria of the respiratory and GI mucosa contain 5% IgE plasma cells vs less than 1% for spleen and peripheral nodes (33).

Human GI tract contains IgM:IgG plasma cell ratio of 5:1. Patients with IgA deficit often have an

increased relative number of IgM plasma cells. Like IgA, IgM polymers can bind SC and be transported across epithelia. The bulk of secretory IgM contains noncovalently bound SC (44).

SOURCE OF SERUM IgA

Serum IgA is present at about 200 mg% with a half life of 6 days vs IgG at 1200 mg% with a half life of 22 days, despite synthesis rates of 36 mg/kg-day and 24 mg/kg-day respectively (30,37).

In humans the origin and role of serum IgA is obscure. It is possible that monomeric and polymeric IgA are synthesized at secretory sites and that the smaller 7S monomer diffuses into serum by way of lymphatics while the 10S dimer is moved onto the epithelial surface by the preferential binding of SC for dimer IgA and intraepithelial transport (44). Alternatively fluorescent antibody studies show greater than 40% of the immunoglobulin containing cells in bone marrow were IgA class (as yet the extent of marrow IgA synthesis is unmeasured) (38).

GALT

The gut associated lymphoid tissue consists of the MLN, PP, and the innumerable lymphoid follicles

in the lamina propria of the small gut (32). Gut lymphatics drain the lymphoid follicles and PP to the mesenteric nodes which eventually drain to the thoracic duct (32,53,54,55).

Thoracic duct lymph contains two lymphocyte populations distinguished by size, small lymphocytes, and large lymphoblasts. Radiolabeling experiments in mice, rats, and rabbits, show the small cells disperse among all lymphoid tissues while the lymphoblasts concentrate in small gut lamina propria, with a few localizing in the spleen, and essentially none in peripheral nodes (32,53,54,55). This suggests the possibility that lymphoblasts originated in the GALT and that these cells (or progeny) migrate to secretory organs where they give rise to dimeric IgA secreting plasma cells in the lamina propria (53,54,55).

Preferential migration of PP lymphocytes to spleen and small gut lamina propria and of MLN lymphocytes primarily to small gut has been demonstrated in animal models by the injection of radiolabeled cells (32,53,54,55).

LYMPHOCYTE MEETS ANTIGEN

Antigen reaches B and T lymphocytes in PP or bronchial associated lymphoid tissue (BALT) through microfold (M) cells, heavily vacuolated specialized epithelial cells lacking microvilli, overlying the PP (32,33).

Precommitted antigen specific cells begin their migration entering afferent lymphatics, travel to MLN or bronchial nodes and thereby to the thoracic duct circulation and finally seed secretory tissue lamina propria throughout the body whether or not antigen is present there (32,33,53,54,55).

Local antigenic stimulation triggers an anamnestic lymphocyte proliferation and increased sIgA production. The response is highly T cell dependent, and in certain cases of IgA deficiency the defect may be in the helper Ts (56). Observations that IgA production is absent in the athymic 'nude' mouse, and that neonatal thymectomy markedly reduces IgA secretion, imply T cell help is crucial for production of IgA (57).

REGULATION OF IgA PRODUCTION

Suppressor Ts in the peripheral blood constitute 20% of circulating T cells, most carry surface receptor for the Fc portion of IgG (TG cells) and most of these regulate IgM and IgG production by secretion of soluble immune response factors that regulate helper Ts in a manner which may or may not be antigen or immunoglobulin class specific (58,59,60). TG cells may effect helper subsets needed for B cell differentiation to IgA plasma cells. Six percent of peripheral lymphocytes are TA but whether they function as helpers or suppressors is unknown (58,59,60). TG and TA cells may represent different stages of differentiation akin to B cell stages.

LOCAL IMMUNITY

sIgA titers in local secretions are better correlated with resistance to viral infections such as polio in the gut and rhinovirus in the nasopharynx than are serum titers (61).

Viral replication in the mucosa of the respiratory or GI tract may remain local with rhinovirus, RSV, myxovirus, and adenovirus, but with polio, echo and measles virus subsequent systemic infection occurs unless serum immunoglobulin is present to neutralize the viremia (61).

Salk vaccine, killed polio virus systemically administered, results in effective immunity via systemic IgG without secretory response, but permits persistant virus carriage on the GI mucosa (61).

Sabin live oral vaccine induces both secretory and systemic immunity and prevents the establishment of a carrier state (61).

Dissociation of systemic and local immunity occurs in RSV infection. Despite specific transplacentally aquired maternal IgG, RSV is a frequent cause of infantile bronchiolitis. Parenteral inactivated vaccine generates high IgG iters without increased protection, and may result in more serious disease secondary to the transudation of complement fixing IgG onto local sites of RSV multiplication (62).

s IgA ACTIONS

Low complement levels, small numbers of phagocytes in mucosal secretions, inability of sIgA to fix complement except by the alternative pathway and its poor opsonic ability are consistent with a strategy of avoiding an inflamatory reaction on mucosal surfaces. If the sIgA barrier is penetrated IgG and IgM will be encountered in the lamina propria and significant tissue damage initiated, as in some colitis (63).

sIgA, mechanical factors, and other agents in secretions appear to act by neutralizing virus, exotoxins, and preventing the adhesion of pathogens to mucosal surfaces (3). Mucous blanket flow, cilial beating in tracheal epithelium, bowel peristalsis, fluid flow, and epithelial desquamation coupled with sIgA binding bacterial surface antigens, all act to inhibit adherence and colonization in infections with pathogens like, cholera, salmonella, and streptococci (3,8).

sIgA from parotid secretions selectively inhibits attatchment of certain oral streptococcal strains to buccal epithelia (8,12). Cariogenic strains of S. mutans produce a dextran from sucrose that somehow allows the bacteria to adhere to the smooth surfaces of teeth. sIgA in the saliva of rats orally immunized by feeding with S. mutans inhibits adherence by directly blocking surface structures and by inhibiting glucosyl-transferases, thereby dextran production (64). A great

deal of work has been done on the development of a carries vaccine (64).

Local immune reactions provide a means of limiting gut and respiratory tract absorption of intact macromolecules by forming nonabsorbable macromolecular complexes that are degraded by bronchial and gut enzymes. Patients with sIgA deficiency have high serum IgM and IgG titers against milk and other food antigens, presumably due to their uncontrolled absorption (63,65). Circulating immune complexes with exogenous antigens and the continuous absorption of macromolecules possibly cross reactive with self antigens, may help explain the clinical association of IgA deficiency and some autoimmune disease (63,66,67).

Antigen specific suppressor T cells migrate from PP to the spleens of enterically immunized animals where they produce soluble suppressor factors (68,69,70). Under certain conditions enteric immunization induces specific systemic non reactivity to antigen, oral tolerance including the abbrogation of contact sensitivity, classically known as the Sulzberger-Chase phenomenon (68, 69,70,71).

IgA DEFICIENCY

DEFINITIONS

sIgA concentration undergoes diurnal variation, varying with site, flow rate, and composition of secretion. In screening for IgA deficiency both serum and secretory levels are assayed. For convenience most studies have assayed salivary sIgA (31).

Normal infants have 21±13 mg% serum IgA, while adult values are 10 times that at 200±61 mg% (29). Serum IgA levels consistantly 2 standard deviations below these values constitute deficiency.

In all humoral deficiencies clinically described there is detectable immunoglobulin, sometimes trace, implying that structural gene deletion is rare (60).

Many asymptomatic adults have serum levels in the 25-75 mg% range (possibly compensitory mechanisms such as increased sIgM levels are responsible).

All symptoms, signs, and disease associations of IgA deficiency are found with serum levels below 5 mg%, even though there are rare individuals with near absent levels who are otherwise normal (29,72,73).

SELECTIVE IgA DEFICIENCY (SIgAD)

Selective IgA deficiency refers to the state where: (74,75)

1) Serum IgA levels are persistantly below 5 mg%.
2) Levels of IgM, IgG are normal or elevated.
3) Repeated measurement of salivary sIgA at the same time of day are less than 2 mg%.

IgE levels are usually found to be normal

3% of screened populations have normal sIgA but documented serum IgA deficiency and are asymptomatic (74). Rare patients with normal serum IgA levels but sIgA deficiency will be brought to attention by clinical symptoms and signs (75).

Selective IgA deficiency as defined here has been detected with a prevalence of 1/350-1/3040 depending on the survey, with a median value of 1/500, making it the most common permanent immunodeficiency state (76).

Five variations of selective IgA deficiency are commonly noted: (67,77,78,79)

1) In asymptomatic persons compensating via unknown mechanisms.
2) In asymptomatic persons compensating with sIgM.
3) Patients with symptoms, signs attributed to the deficiency.
4) 5-20% (excluding those with combined immunodeficiencies) will have T cell function anomalies.
5) Up to 20% of those symptomatic in categories 3) and 4) have other diseases with frequency of

occurance so low that chance association is unlikely e.g. atopic disease, recurrent respiratory disease (bronchitis, bronchiectasis, recurrent pneumonia, pulmonary hemosiderosis, GI and nervous system disease, malignancy, and most commonly, autoimmune diseases, accounting for 50% of the total.

Hypogammaglobulinemia is common in families of SIgAD patients with both autosomal dominant and recessive inheritance reported (73). Many cases are associated with deletions in chromosome 18 or a ring 18, but the finding is inconstant and allotypes of IgA segregate independently (80,81).

Acquired IgA deficiency occurs in patients treated with DPH or penicillamine, is frequently associated with sinopulmonary tract infections, and may return to normal levels when the drugs are discontinued (77,82,83). Recent work suggests that immune disturbances may precede the drug use (84).

Twin studies hint environmental factors during fetal life can cause increased penetrance of the defect in susceptibles. Celiac disease, recurrent URI, and IgA deficiency were reported in one monozygous twin, and selective IgA deficiency has been noted in one of HLA identical twins with thyroiditis and diabetes (85).

CLINICAL CHARACTERISTICS OF SIgA DEFICIENCY

Viral and bacterial infections of the upper and lower respiratory tract, middle ear, and GI tract are especially common in SIgAD children (86).

Recurrent sinopulmonary disease is the most common presentation overall, with an occasional patient coming to attention because of recurrent or chronic right middle lobe pneumonia (77,86). Pulmonary hemosiderosis is more common than expected (77,86).

The prevalence of SIgAD among atopics is 1/400-1/200 vs 1/800-1/600 in normals (63,65,68). The reduction in serum IgA may result in decreased competition for antigen increasing the likelihood of combining with IgE. The decreased sIgA may result in absorption of near intact proteins and increase the likelihood of sensitization.

Increased prevalence of antibody to bovine proteins, and circulating immune complexes including bovine immunoglobulins have been noted in sIgA deficiency (65). Removal of bovine products from the diet hasn't been clearly associated with clinical improvement.

30-40% of SIgAD patients have serum anti IgA antibodies,most without history of blood product administration (39,40,66). A few may develop anaphylactic reactions following parenteral administration of blood products. Sensitization may have occured during breast

feeding in infancy, transplacental maternal IgA transfer or cross reaction with bovine immunoglobulin (63,65).

Celiac disease, ulcerative colitis and regional enteritis are over represented among these patients (87).

Systemic lupus erythematosis, rheumatoid arthritis, dermatomyositis, thyroiditis, pernicious anemia, Sjogren's syndrome, and chronic active hepatitis are all occasionally seen in association with SIgAD but only the increased prevalence of SIgD in SLE and RA is statistically significant. The clinical presentations and presence of characteristic antibodies in these diseases do not significantly differ in those patients with IgA deficiency (67,78,79,88).

SIgAD has been reported in patients with reticulum cell sarcoma, squamous cell carcinoma of lung and esophagus, and thymoma, several cases with associated autoimmune disease and recurrent infection (67).

PATHOGENESIS

In a few cases inappropriate suppressor T cell activity results in SIgAD. B cells isolated from Ts and exposed to polyclonal B cell activators (like pokeweed mitogen) will secrete IgA (60).

Some investigators have found cases of intrinsic B_A cell defects, others have noted defects in T helper cell activity, and still others have found combinations of these defects (56,58,59,60). Non-

specific T cell defects are present in 10% of patients with SIgAD (56,58,59,60). The association of autoimmune disease and SIgA deficiency may be due to a shared disturbance in T cell function.

TREATMENT

Currently there is no effective treatment for selective IgA deficiency other than direct antibody replacement on mucosal surfaces in the form of colostrum (1, 2,3,4,5). Eye drops, ear drops, nose drops, or ingested colostrum have all been used to treat particular infectious complications of SIgA deficiency with good success (1,2,3,4,5).

Colostrum is the high sIgA content, 1000 mg%, post partum product of the mammary glands. Ingesting a colostrum bolus provides the neonate with specific passive immunity of days duration to gut pathogens in the mothers environment (1).

It has been demonstrated that maternal enteric immunization increases the titer of specific sIgA antibody in colostrum and milk, and enteric vaccination of pregnant women to ensure their colostrum contains protective sIgA titers against gut organisms that cause epidemic disease in neonates has been suggested (1).

PRODUCING HYBRIDOMAS SECRETING DIMERIC IgA (+J)

In a typical attempt to produce murine hybridomas one lymphocyte per two or three hundred thousand undergoes successful fusion. The number of antigen specific hybridomas produced is directly correlated with the number of B cell blasts (size determined) at fusion (20,89).

Using spleen as a lymphocyte source and conventional parenteral immunization techniques, less than 1% of hybrids produced will secrete IgA (90). These low yields encourage the investigation of alternate techniques of immunization and sources of lymphocytes.

ORAL (ENTERIC) IMMUNIZATION

Andre et al have found primary orogastric immunization of BALB/c mice (the strain often used in hybridoma work) with sheep red blood cells on four consecutive days produces a biphasic delayed peak response of 100 IgA indirect plaque forming cells (IPFC) and 50 IgM PFC per million gut lymphocytes after 14 days (91).

Second immunizations for four consecutive days at 14 and 90 days induced earlier peak anamnestic responses of 250 IgA IPFC and 150 IgM PFC per million at 23 days and 450 IgA IPFC and 300 IgM PFC per million at 94 days (91).

In an earlier study they noted oral immunization induced IgA IPFC and IgM PFC in the spleen, but second

immunization did not induce a secondary response there (92).

In this experiment immunization immunization induced greater and prolonged biphasic primary delayed peak response in the gut, suggesting that IPFC and PFC detected previously in the spleen were gut lymphocytes on their way back to the gut.

Second immunization yielded increased gut response without any splenic response, secondary to tolerance or because the gut cells didn't recirculate. These findings suggest the absence of splenic response occurs because the memory cells don't recirculate but are retained in the gut (91,92).

Plasma immunoglobulin response did not differ in primed and boostered animals, concurring with previous observations on the poor correlation of circulating antibody levels and local immunity (91).

These observations imply fusions using splenic lymphocytes containing specific IgA IPFC after primary intragastric immunization might produce specific dimeric IgA producing hybrids.

Since intragastric boostering produces no secondary splenic response, fusions using spleens from boostered mice are paradoxically less likely to produce specific IgA secreting hybridomas than using spleens from primarily immunized animals.

The gut limited IgA anamnestic response to intragastric immunization and boostering observed in these experiments implies fusions using GALT may result in high frequencies of specific dimeric IgA secreting hybrids.

THE NATURE OF THE LARGE DIVIDING CELLS IN THE GALT

Guy-Grand et al studied suspensions of murine (BALB/c) lymphocytes from peripheral lymph node (PLN), Peyer's pathches (PP), mesenteric lymph node (MLN), and thoracic duct lymph (TDL) (53). Cells were incubated in vitro with tritiated thymidine (to identify blasts), followed by immunofluorescent staining and autoradiography. Their results may be summarized:

	%blast	%blast Bblast	%Bblast with surface IgA	%blast with surface IgA	%Bblast with internal IgA	%blast with internal IgA	%blast with surface Ig
PLN	2.6	61	3	0.5	0.4	0.06	
PP	4.2	77	38	1.2	0.9	0.3	2.2
MLN	1.7	45	13.7	0.1	6.6	0.5	0.22
TDL	0.5	33	27	0.4	16	0.3	

PP were found to be an especially rich source of blasts bearing surface IgA. PP, MLN, TDL demonstrated approximately equal % IgA containing blasts. All cells with internal IgA (iIgA) also displayed surface IgA. PLN was an especially poor source of iIgA blasts.

20 hours after injection of tritiated thymidine labeled lymphocytes from these tissues they observed the results summarized:

	% Label Density Found in Spleen			
SOURCE	PLN	MLN	PP	GUT
PLN	64	25	7	1
PP	1	5	14	5
MLN	34	90	62	40
TDL	8.4	43	83	630

PP blasts homed mainly to spleen, while those from MLN and TDL accumulated in the intestinal mucosa (small bowel concentration 8 times large bowel) and PP. The number of IgA plasma cells found in the gut lamina propria was directly proportional to the number of donor blasts containing iIgA. Apparently it is the iIgA blasts which home to gut to become IgA plasma cells.

Craig and Cebra's observation that transfer of PP lymphocytes into lethally irradiated rabbits leads to the appearance of labeled IgA plasma cells in spleen and gut might be explained by maturation of the dividing transfered cells to permit increased iIgA synthesis (32,55). It is concievable that while acquiring the capacity to synthesize iIgA B_A blasts shift to producing dimeric IgA, and that dimer or J chain on the cell membrane somehow interacts with SC to cause the homing effect (55).

These experiments imply PP and MLN would be optimal lymphocyte sources for generating dimeric IgA secreting hybridomas if enough cells could be harvested under aseptic conditions from a properly immunized and boostered mouse.

AN INTERESTING ANTIGEN, LIPOTEICHOIC ACID

Teichoic acids (TA) are substituted polymers of glycerol or ribitol phosphates in the cell walls and membranes of gram positive bacteria (93,94).

Cell wall TAs are covalently linked to the peptidoglycan of some bacterial cell walls under most growth conditions. Studies in several bacteria indicate that wall TA is located sufficiently close to the surface for specific antibody reactions to occur (95).

Membrane TAs, lipoteichoic acids (LTA), are a more characteristic component of gram positive bacteria, and not as dependent on growth conditions as the wall polymers (94). LTAs are polymers of 1-3 phosphodiester linked glycerolphosphate residues (typically 25-30), the backbone, variously substituted with glycosyl and D-alanyl esters, and covalently bound to a glycolipid (identical to the free glycolipid of the plasmalemma, primarily palmitic acid) through a phosphodiester bond between a sugar hydroxyl of the glycolipid and the terminal residue of the TA (total MW 10,000) (94).

LTAs of some organisms provolk serological reactions. The group antigen of the group D streptococci is an LTA (95). IgM, IgG, and IgA humoral responses have been noted.

Wicken et al propose a wall-membrane model to

explain these results (94,96). LTA molecules are envisioned as imbedded in the plasma membrane by their hydrophobic tails, while their polar glycerol-phosphate chains are intercalated in the polysaccharide peptidoglycan network of the cell wall. Depending on cell wall thickness, peptidoglycan cross linking, the number of residues in the polar chain and its conformation in the cell wall, the distal residues could behave as surface antigens. By labeling thin sections of bacteria with backbone specific rabbit IgG then with ferritin labeled goat anti rabbit IgG, they observed label extending from the cell membrane through the cell wall and in some cases beyond.

RANTZ ANTIGENS

LTAs will directly sensitize erythrocytes because their lipid moiety can dissolve in the mammalian cell membrane. Hemagglutination may be used to detect low concentrations of LTA and or antibody to LTA (93,94,95).

Rantz antigens, erythrocyte sensitizing antigens obtained from saline washings or culture fluid of gram positive bacteria are presumed to be LTAs (93). It has been noted that culture fluid from oral streptococci and lactobacilli may contain up to 50 micrograms/ml of LTA, an amount comparable to that in the cell mass. Whether this is evidence LTA is secreted, or results from turnover of cell wall or membrane is unknown (97).

ANTI BACKBONE

In most cases when anti LTAs have been detected in human serum they have been directed against the polyglycerolphosphate backbone (94,95,96). The degree of substitution determines whether the antibodies are backbone or substituent specific (95). Antibodies to backbone seemingly account for the observations of a common gram positive antigen (93,95).

Group specific anti LTAs from group D and N streptococci and group F lactobacilli are substituent specific (95). Anti L. casei LTA (unsubstituted) is naturally backbone specific and cross reacts with a variety of LTAs (94,95,96). Substituted LTAs may also generate backbone specific antibodies (95). It is possible that one organism may act as a primary stimulus for the production of backbone antibody and that a variety of organisms may provide subsequent stimuli.

CROSS REACTIVITY WITH MAMMALIAN ANTIGENS

Wicken et al speculate anti LTA in humans may cross react with cell constituents and participate in inflamatory sequelae to streptococcal infections like rheumatic fever or glomerulonephritis (95,96).

Cross reaction with complement fixation by anti backbone with cardiolipin (diphosphatidylglycerol)

could explain biological false positive syphilis tests (99).

Beachey et al demonstrated LTA is a T cell mitogen, and that although LPS and LTA are both amphipathic molecules binding via lipid tails to mammalian lymphocyte membranes, LTA selectively acts as a T cell mitogen while LPS selectively stimulates B cells (100).

Studies in which parenteral administration of LTA is used to induce experimental nephritis, delayed hypersensitivity, and experimental arthritis are currently under way (101).

Lafer et al studied the diversity of antibodies produced in systemic lupus erythematosis (SLE), especially those reacting with nucleic acids, native or denatured DNA and RNA, nucleoproteins, cell surface antigens, cardiolipin, and anticoagulant factors (102). Antibodies of these sorts were often reactive with the sugar phosphate nucleic acid backbone, phosphodiester linkages separated by three carbons of adjacent sugars (102). These structural features are also found in cardiolipin and the backbone of LTA, and may explain the false biological syphilis tests seen in SLE (102,103).

LTA AND ADHERENCE TO EPITHELIA

Bacterial adherence is prerequisite for colonization and invasion of mucosal surfaces.

Ofek et al studied adherence of group A streptococci to buccal epithelial cells in vitro (3,10,104).

They demonstrated both LTA and its lipid moiety prevented the adherence of streptococci to preincubated epithelial cells. Streptococci preincubated with anti LTA lost the ability to adhere to epithelial cells. They further showed that adherence inhibitory effects of antisera directed against whole group A bacteria was attributable to the presence of anti LTA titers. They conjectured the binding of LTA via its lipid tail to mammalian cell membranes may play the central role in the binding of gram positive pathogens to mucosal surfaces.

Recent work by Beachey et al demonstrated that the lipid moiety of LTA exposed in protein (largely M protein) LTA complexes on the surface of group A steptococci is the adhesin mediating attatchment of the organisms to the lipid binding sites of fibronectin (the receptor) on human cell membranes (105). It was shown that the number of streptococci capable of adhering is directly proportional to the fibronectin present on epithelial cells, and that preincubation of streptococci with fibronectin inhibits adhesion.

Ofek and Beachey studied the relation of epithelial binding and surface fimbriae of streptococci (11). Previous observations by Ellen and Gibbons suggested fimbriae were intimately involved in the binding to mucosa (3,104,106). Swanson et al had demonstrated an association of the antiphagocytic M protein and these fimbriae (106). It was later noted by Ellen and Gibbons that M protein producing streptococcal strains adhere to epithelia while M negative strains (later shown lacking fimbriae) do not (104). Epidemiologic studies consistently demonstrate more than half the streptococcal strains isolated from humans lack M antigens (107).

Ofek and Beachey found organisms treated with pepsin at pH 5.8 retained their fimbriae and mucosal binding ability though they lost their M protein and resistance to phagocytosis (11).

Dissociation of resistance to phagocytosis and epithelial binding ability has been reported in piliated gonococci and piliated Proteus mirabilis, suggesting the distinction between these virulence mechanisms may be a more general phenomenon among pathogens.

Using the in vitro adherence assay they showed streptococcal binding to treated cells was inhibited by LTA, but not by M protein, C carbohydrate, peptidoglycan, or lipopolysaccharide (11). As before adherence was blocked by preincubating the bacteria with anti LTA.

Further work by Alkan, Ofek, and Beachey demonstrated LTA inhibited binding of streptococci to skin as well as

oral epithelia (12).

Rolla et al have proposed LTA might be a crucial element in the adherence of sucrose grown Streptococcus mutans to dental enamel by charge interaction between the numerous anionic backbone phosphate groups and enamel (64). LTA trapped in the polysaccharide coat of S. mutans accounts for the threefold increase in cell bound LTA when 5% sucrose is substituted for 5% glucose in the culture medium. Further, sucrose grown S. mutans has twice the affinity of glucose grown strains for hydroxyapatite (HA), and behaves as negatively charged molecules with respect to HA in the presence of competing ions.

RECENT RELATED WORK OF OTHER INVESTIGATORS

A year into the current work two reports of related investigations appeared.

Colwell et al were able to generate high numbers of anti trinitrophenol (TNP) polymeric IgA secreting hybridomas by priming germ free BALB/c mice with sheep red blood cells (SRBC) via intragastric intubation on two consecutive days followed one week later by intubation with TNP haptenated SRBC, after 7 days spleen cells were fused with the non secreting murine myeloma (X63.Ag8.653) (108). 65.4% of hybrids produced anti TNP, 54% of IgA class, with the remainder equally distributed between IgM and IgG. They report greater than 90% secreted IgA was polymeric.

They have also produced hybridomas secreting IgA specific to surface determinants of an etiologic agent of human dental carries, the gram positive S. mutans.

The success of their work is consistant with Andre et als observations on the development of IgA IPFC in the spleens of mice as part of the primary response to intragastric immunization (as previously noted).

Dean et al were able to produce a rat hybridoma secreting cell surface antigen specific polymeric IgA by fusing MLN cells from rats immunized by direct injections of allogenic cells into PP at a 14 day

interval. Two days after the second injection MLN cells were fused with a rat myeloma (Y3.Ag.1.2.3) (109). Presumably antigen leaking into MLN afferent lymphatics triggers an anamnestic blastogenic response there. The polymeric nature of the IgA was demonstrated by the kinetics of the disappearance of radiolabeled antibody from the blood and subsequent reappearance in the bile (via transhepatic transport) after IV injection of a rat.

A good idea often independently occurs to many people at about the same time.

MURINE DIMERIC IgA ANTI BACKBONE AS A POTENTIAL CLINICAL AGENT

sIgAs function on mucosal surfaces to prevent the adherence of pathogens. They are poor opsonins, and do not activate complement except by the alternate pathway.

Dimeric IgA (+J) will stabilize by binding to free SC in mucosal secretions, and it has been shown human SC has considerable affinity for heterologous polymeric antibodies including murine polymeric IgA (+J)s.

Hybridoma technology may soon provide the ability to produce limitless quantities of human or heterologous specific dimeric IgA for use as specific passive mucosal immune agents, in effect made to order super colostrums.

In principle MLN or PP lymphocytes aseptically harvested from enterically immunized and boostered mice, or splenic lymphocytes from recently immunized but unboostered mice displaying anamnestic blastogenic response might be fused with log phase cells of a non secreting murine myeloma to generate specific dimeric IgA secreting hybridomas.

LTA has been demonstrated to be the adhesin of the group A streptococci and possibly many other gram positive epithelial pathogens.

Preincubation of streptococci with backbone specific antibody has been shown to block adherence to human buccal epithelial cells in vitro.

The polyglycerolphosphate backbone common to all LTAs is a 25-30 residue polymer affording many identical binding sites to a single specificity (monoclonal) antibody.

Murine hybridoma derived dimeric IgA anti LTA backbone will likely have considerable ability to block adherence of streptococcal pathogens in vitro.

In vivo it may be useful as a safe clinical agent providing passive mucosal immunity against a wide variety of gram positive pathogens, and might indeed be clinically employed as a super colostrum.

A step toward this goal is demonstrating IgA secreting murine hybridomas can be produced using GALT from enterically immunized and boostered mice.

MEDIA

Media required in the fusion protocol were prepared as follows:

100 x HT

38.8 mg Thymidine MW 242.2 (Sigma)

136.1 mg Hypoxanthine MW 136.1 (Sigma)

100.0 ml DD H_2O warmed to 80-90 C

filter sterilized and stored at -20 C in 5 ml aliquots

100 x A

35.2 mg Aminopterin MW 440.4 (Sigma)

100.0 ml DD H_2O

filter sterilized and stored at -20 C in 0.5 ml aliquots

8-Azaguanine

200.0 mg Azaguanine MW 152.1 (Sigma)

10.0 ml DD H_2O

dissolved by adding 1N NaOH and brought up to 20 ml with DD H_2O, filter sterilized and stored at -20 C in 1 ml aliquots

MEDIUM FOR SP2

500 ml RPMI 1640 (Gibco)

5 ml HEPES (Gibco) 1M

50 ml Calf serum heat inactivated in 56 C water bath 45 minutes

10 ml Penicillin 5000 IU/ml-Streptomycin 5000 mcg/ml (Flow)

1 ml Azaguanine

HT MEDIUM

500 ml DMEM with 4500 mg/l glucose (Gibco)
5 ml HEPES
5 ml Sodium pyruvate (Flow 100mM)
5 ml HT
50 ml NCTC 109 (Microbiological Associates)
100 ml Fetal calf serum heat inactivated
5 ml Antibiotic-antimycotic (Gibco)
Penicillin 10,000 IU/ml
Streptomycin 10,000 IU/ml
Amphotericin 25 mcg/ml

HAT MEDIUM

HT medium
0.5 ml 100 x Aminopterin

50% POLYETHYLENE GLYCOL 1500 (BDH-Gallard Schlesinger)

20 g PEG Autoclaved 30' in a 100 ml bottle
20 ml DMEM added to cooling but still molten PEG
stored at room temperature in 1 ml aliquots

SP2 myeloma kindly provided by Dr. Kim Bottomly

All cultures were incubated in 8% CO_2 at 37 C

MATERIALS AND METHODS

Immunization Schedule

On two four consecutive day periods three weeks apart 5 mice fasted 8 hours were placed in a saturated diethylether atmosphere at room temperature for twenty seconds, restrained and orogastrically intubated to 6 cm with a #20 polyethylene tube (Intramedic 0.011" inner x 0.024" outer diameter) and 0.2 ml of Freund's complete adjuvant delivered from a tuberculin syringe abutted to the tubing by a #27 needle.

Source of Lymphocytes

On the final day mice fasted 8 hours were killed by cervical dislocation. The peritoneal cavity was immediately aseptically opened and mesenteric lymph nodes dissected out from cecum to the root of the mesentery as a single mass and placed in a small sterile Petri dish containing 5 ml sterile Hanks Balanced Saline Solution (HBSS, Difco) warmed to 37 C.

The bowel was fanned out and approximately 10 Peyer's patches removed with microdissecting scissors from the serosal side of the ileal antimesenteric border without entering the bowel and placed in a separate sterile Petri dish containing 5 ml sterile

warmed HBSS.

Pooled tissues in the MLN and PP dishes were teased apart with sterile needles, debris allowed to settle for one minute, and the lymphocyte rich supernatants removed with sterile Pasteur pipettes.

Expected recoveries are 40 million and 10 million lymphocytes respectively, roughly half that of a single spleen.

Fusion

On the day before fusion SP2 cells were transfered to azaguanine free medium to deplete intracellular pools and PEG was placed in an 8% CO_2 incubator to adjust pH.

SP2 cells washed in 37 C DMEM and freshly harvested lymphocytes were mixed 3:1 in a 15 ml centrifuge tube and spun at 1,500 rpm for 10', supernatant removed with pellet intact. 0.8 ml PEG was added to the pellet over 1', mixing gently with the pipette tip and the tube hand warmed 1'. 1 ml of DMEM was added over 1' and the tube again hand warmed 1' before adding 10 ml DMEM over 2' and spinning at 1,500 rpm 10' then removing the supernatant. The pellet was resuspended in HAT medium to 10-100 million cells/ml and .1 ml added to each of the inner 60 wells of 96 well microtiter plates, the outer 36 wells of which were prefilled

with a saturated aqueous copper sulfate solution.

On the 4th and 7th days post fusion half the supernatant from each inner well was aspirated with a Pasteur pipette and replaced with .1 ml HAT medium, then fusomas were fed HT medium at 3-5 day intervals.

Clones were macroscopically evident at 10-20 days.

ELISA for Murine Antibody Class

200 microliters of 1:20 borate buffered saline (BBS, pH 8.4) dilution of 1mg/ml rabbit anti mouse immunoglobulin (Bionetics) was added to each well of a polyvinyl chloride microtiter tray (Linbro) and incubated overnight at 4 C. The tray was washed 5 times in phosphate buffered saline (PBS, pH 7.4) then blocked by adding 200 microliters of 10% bovine serum albumin (Gibco) in BBS, incubating 4 hours at room temperature and again washing 5 times with PBS.

100 microliters of 1:1 BBS diluted supernatant was added to each of the inner 60 wells, incubated 4 hours at room temperature and washed 5 times with PBS.

100 microliters of 1:200 BBS diluted 1 mg/ml alkaline phosphatase conjugated goat anti mouse immunoglobulin (Bionetics) was placed in all wells, incubated 4 hours at room temperature and washed 5 times with PBS.

100 microliters of a 0.1% solution of p-nitrophenyl-phosphate disodium in diethenolamine buffer (pH 9.8) was

added to all wells, incubated 2 hours at room temperature, and reaction terminated by the addition of 100 microliters 1N NaOH to all wells. Absorbance at 405 nm was measured with a Titertek multiscan photometer (Flow).

Positive wells were assayed for antibody class as above by using 1:500 BBS dilutions of 1 mg/ml alkaline phosphatase conjugated goat anti mouse IgA, IgG, and IgM respectively.

Protocol

Two separate groups of five female 12 week old BALB/c mice (Jackson) were immunized and boostered with undiluted Freund's complete adjuvant as above.

Lymphocytes were harvested from MLN and PP as above with the recovery of 37.5 million MLN and 10.5 million PP lymphocytes from group I and 30.0 million MLN and 12.3 million PP lymphocytes from group II, separately fused with SP2 myeloma cells as above, and plated in 120 master microtiter wells for each of the four categories.

At 20 days post hybridization growth was determined by observation with an inverted microscope and supernatants collected and stored frozen at -70 C for 2 years before assay by ELISA as above.

RESULTS

	Group I		Group II		Totals	
	MLN	PP	MLN	PP	MLN	PP
Lymphocytes($x10^6$)	37.5	10.5	30.0	12.3	67.5	22.8
Wells cultured	120	120	120	120	120	120
Wells contaminated	0	0	0	60	0	60
Growing wells	3	22	9	13	12	35
Frequency($x10^{+6}$)	12.5	0.5	3.3	0.5	5.6	0.5
Ig positive	0	7	0	3	0	10
IgA	0	2	0	2	0	4
IgG	0	2	0	0	0	2
IgM	0	3	0	1	0	4

Absorbance at 405 nm on ELISA for:

Well	IgA	IgG	IgM
IP1D6	.137	.355	1.047
IP1E7	.095	.603	.113
IP2B8	.110	.207	.731
IP2C7	.088	.615	.105
IP2C10	.357	.211	.104
IP2D8	.125	.238	.772
IP2E6	.338	.197	.101
IIP1B6	.339	.235	.108
IIP1C8	.109	.221	.652
IIP1E5	.303	.224	.108

The large percentage of nongrowing wells on all plates implies a high probability of monoclonality (90%) in growing wells. Monoclonal origin of growing cultures is assumed (see Appendix).

The optical density data obtained by ELISA of the supernatants is unambiguous despite the relatively poor specificity displayed by the alkaline phosphatase conjugated goat anti mouse IgG.

Of note is the low hybridization frequency of MLN lymphocytes with SP2, 1 per 5.6 million and the lack of detectable immunoglobulin secretion of all 12 hybrids obtained.

PP lymphocytes fused with SP2 cells produced hybridomas at a frequency of 1 per 470 thousand, 10 of the 35 (29%) were assayed as immunoglobulin secreting with IgA, IgG, and IgM classes represented 4,2, and 4 times respectively.

The data indicate PP lymphocytes from an enterically immunized and boostered animal are more likely to hybridize than MLN lymphocytes with SP2 cells in this protocol, $X^2_c(1df)=85$, P=.001, and that these PP hybrids have a higher frequency of immunoglobulin production than the MLN hybrids produced, $X^2_c(1df)=2.8$, P=.10.

Antibody specificity was not investigated, nor was an attempt made to assay IgA containing supernatants for polymeric antibody.

DISCUSSION

It was demonstrated that MLN and PP from enterically immunized and boostered BALB/c mice can be harvested under aseptic conditions with average recoveries of 7 and 2 million cells per animal respectively, quantities useful for the study of cell surface and internal immunoglobulin class frequencies.

Pooled tissue from many animals was required to accumulate enough lymphocytes for fusion with SP2 myeloma cells to successfully yield immunoglobulin secreting hybridomas.

MLN lymphocytes were shown to be poor fusion partners with a hybridization frequency 1/10 that of PP and splenic lymphocytes with SP2 cells. The significant relative paucity of MLN hybrids might stem from one or more causes including:

a) Differences in handling of the MLN and PP SP2 fusomas. The MLN SP2 post fusion mix was plated at 3 times the cell density of the PP SP2 mix.

Increasing cell density in the 1-10 million/ml range typically does not effect or slightly increases the frequency of recovery of splenic SP2 hybrids, and unless MLN SP2 fusomas behave quite differently, the cell density disparity of the cultures is unlikely to be critical.

b) Differences in the B blast quantity or quality

in the parent tissues.

Stahli et al demonstrated hybridization frequency is directly proportional to the blast (identified by cell volume) concentration of the splenic parent lymphocytes (89).

If the concentration of B lymphoblasts present in MLN is significantly different from that in PP or if these MLN blasts are at a different stage of differentiation less adept at forming hybridomas the above discrepancy in frequencies is explicable.

Guy-Grand et al observed a PP:MLN ratio of B cell blasts (identified by tritiated thymidine uptake and the presence of surface immunoglobulin) in unimmunized BALB/c mice of approximately 10:1 (53).

If it can be assumed that the definition of blast by criteria based on cell volume identifies the same set of cells as tritiated thymidine uptake and surface immunoglobulin, then a simple difference in B blast concentration in PP and MLN is a plausible explanation for the poor showing of the MLN lymphocytes.

Perhaps the difference in blast concentration of MLN and PP might be explained by Andre et al's hypothesis concerning their observation that intragastrically immunized BALB/c mice display a primary gut and splenic (systemic) immune response, but that boostering fails to elicit a secondary anamnestic reponse outside the gut lamina propria (91,92). They conjecture primary

gut immunoblasts travel from gut to MLN to thoracic duct to bloodstream and finally settle in gland or gut lamina propria, but that the gut memory lymphoblasts induced by boostering do not travel. The differnce in blast concentration of the MLN and PP might be due to a lack of gut memory blasts in the MLN.

The lack of immunoglobulin production in all 12 MLN hybridomas might be explained by assuming:

a) A chance event. The difference in ability of MLN and PP SP2 hybrids to secrete antibody was statistically insignificant, P=.1 by X^2_c.

b) MLN and PP B blasts are at different stages of differentiation with MLN blasts less inclined to instruct MLN SP2 hybrids in the manufacture and secretion of immunoglobulin.

Guy-Grand et al provide evidence for this maturation argument with their observations on the increased concentration of blasts with internal immunoglobulin in MLN relative to PP, and the homing characteristics of radiolabeled B blasts from those tissues injected into mice (53). MLN blasts preferentially home to MLN, PP and gut, while PP blasts prefer the spleen and PP.

These observations and those of Andre et al above hint that shortly after primary enteric immunization spleen, MLN, and PP may all be good sources of immature B blasts capable of fusing with SP2 cells to secrete

immunoglobulin. The success of Colwell et al in generating antigen specific polymeric IgA secreting hybridomas by fusing splenic lymphocytes from intragastrically immunized but unboostered germ free mice with non secreting X63.Ag.8.653 myeloma cells is explicable in light of the above (108).

c) Different ratios of helper and suppressor T cells in MLN and PP in situ at the time of fusion or perhaps even carried over in cell culture might result in differences in the ability of MLN and PP SP2 hybridomas to manufacture and secrete immunoglobulin.

Richman et al have demonstrated enterically induced systemic immune tolerance in mice is due to the post intragastric immunization induction of suppressor T lymphocytes in the gut which travel to spleen, peripheral nodes, and perhaps MLN (70,71).

It is concievable that the failure of the MLN SP2 hybrids to secrete antibody may be another manifestation of T cell enforced enterically induced systemic tolerance.

Failure to detect immunoglobulin in 25 of 35 (71%) of PP SP2 hybridoma supernatants might be explained by:

a) Chance.

Goding points out early after fusion there is a strong tendency for chromosome loss and overgrowth with the metabolically advantaged non secreting mutants of the original clone (20). Subcloning is generally required to preserve secreting cultures.

Collection of supernatants at 20 days post fusion rather than immediately on noting macroscopic growth in the interim was undoubtedly contributory.

b) The existance of PP B blast subsets at different stages of maturation with some less inclined to instruct PP SP2 hybrids in immunoglobulin manufacture and secretion (see above).

The observed ratio of the number of IgA:IgG:IgM secreting hybrids is consistant with the 54:16:30 ratio of PP blasts displaying these surface antibodies respectively as noted by Guy-Grand et al, $X_c^2(2df)=.185$ (53).

Antigen specificities of the immunoglobulins were not investigated. It was assumed intragastric immunization and boostering with Freund's complete adjuvant would result in generalized non specific GALT stimulation.

The ability to direct specificity of antibody secreted by GALT derived hybridomas by enteric immunization with antigen is an implicit assumption of this work.

Colwell et al and Dean et al produced antigen specific secreting hybrids after intragastric immunization of germ free mice and direct injection of antigen into rat PP respectively (108,109).

IgA containing supernatants were not assayed for polymeric product. It is conjectured IgA secreting GALT derived hybrids will preferentially secrete polymeric antibody.

Assay of these supernatants alongside a monomer and polymer murine IgA supernatant from MOPC 315

by SDS electrophoresis, electroblotting, and I^{125} iodinated antibody probe autoradiography is currently in progress (110).

Colwell et al and Dean et al produced IgA secreting hybrids in mice and rats respectively, greater than 90% were found to secrete polymeric antibody (108,109).

This first step toward the design of a protocol for generating GALT derived specific murine dimeric IgA (+J) secreting hybridomas from enterically immunized and boostered animals uncovered several obstacles.

MLN cells were found to be poor fusion partners and the rare MLN SP2 hybrids did not secrete antibody.

The small number of PP lymphocytes recoverable (2 million/BALB/c) require that tissue from 50 animals be pooled to equal the number of cells available from a single spleen.

Supplementing PP with jejunal and ileal lamina propria lymphocytes (10-20 million/BALB/c) could reduce requirements to 10 animals, but it remains to be seen if these lymphocytes can be aseptically recovered and if fusions with SP2 cells produce hybrids with similar characteristics to PP SP2s.

Alternatively, harvesting MLN,PP, and splenic lymphocytes one week after immunization without boostering a la Colwell et al, taking advantage of the circulating lymphoblasts of the primary gut immune response seems particularly attractive since it may be possible to

reduce tissue requirements to a single animal (108).

Once a suitable protocol has been established, animals could be intragastrically immunized with sheep red blood cells (SRBC) sensitized by incubation with an unsubstituted LTA (from L. casei) to ensure anti backbone production (93,94).

Supernatants could be tested for anti LTA activity and IgA class by passive hemagglutination of sensitized SRBC or ELISA (93,94,95,96,111). Anti backbone activity could be verified by VDRL, and the polymeric nature of the antibody established by gel electrophoresis, electroblotting, and I^{125} iodinated antibody probe autoradiography (110). Most important, the functional activity of the antibody could be demonstrated by its ability to block streptococcal adherence to epithelia in vitro (3,10,104).

The clinical potential of specific passive mucosal immunity has barely been gauged largely because specific agents were not available in quantity. That is about to change.

Especially interesting is the recent unpublished work of Colwell et al in producing murine polymeric IgA anti S. mutans secreting hybrids (108).

A little 'mousewash' a day may keep the dentist away.

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APPENDIX

A NEW THEOREM-MURPHY'S LAW OF CLONING BY LIMITING DILUTION

Assuming progenitors independently Poisson distributed with average number x interesting and y uninteresting per culture, the probability of having placed i interesting in culture with j uninteresting is:

$$P(i,j) = P_x(i)\, P_y(j) = e^{-x}\frac{x^i}{i!}\, e^{-y}\frac{y^j}{j!}$$

The probability of an interesting culture being monoclonal is:

$$P_M = \frac{P(1,0)}{1-P_x(0)}$$

Expressed in terms of the fraction of sterile cultures s, and uninteresting (including sterile) u:

$$s \leq P_M = \frac{s \ln u}{u-1} \leq \frac{s \ln s}{s-1} \qquad , \; 0 \leq s \leq u \leq 1$$

Murphy's Law- The probability an interesting culture is monoclonal is bounded below by the fraction of sterile cultures and less than or equal that of a random growing culture. (see Table 1)

TABLE 1

$$P_M = \frac{s \ln u}{u-1}$$

u \ s	0.1	.2	.3	.4	.5	.6	.7	.8	.9	1.0
0.1	26%									
.2	20	40								
.3	17	34	52							
.4	15	31	46	61						
.5	14	28	42	55	69					
.6	13	26	38	51	64	77				
.7	12	24	36	48	59	71	83			
.8	11	22	33	45	56	67	78	89		
.9	11	21	32	42	53	63	74	84	95	
1.0	10	20	30	40	50	60	70	80	90	100

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